American Journal of Hematology 58:285–292 (1998)

Structural Characterization and Functional Effects of a Circulating Heparan Sulfate in a Patient With Hepatocellular Carcinoma

David S. Wages,[1]* Ilona Staprans,[2] Julie Hambleton,[3] Nathan M. Bass,[3,4] and Laurence Corash[1]

[1]Department of Laboratory Medicine, University of California San Francisco, San Francisco, California
[2]The Department of Veteran's Affairs Medical Center, San Francisco, California
[3]Department of Medicine, University of California San Francisco, San Francisco, California
[4]The Liver Center, University of California San Francisco, San Francisco, California

A circulating anticoagulant was isolated from the plasma of a 42-year-old man with cirrhosis and hepatocellular carcinoma who had an unusual coagulation test profile. The patient developed a fatal coagulopathy, unresponsive to protamine therapy or plasma exchange following liver biopsy. However, at presentation, routine hemostasis assays were normal. The patient had mucocutaneous bleeding but the sole laboratory abnormality was a prolonged thrombin time (TT = 99 s, normal 25–35 s). Protamine titration indicated activity equivalent to a heparin concentration of 6–7 U/ml. Antithrombin III (AT III) antigen and activity were markedly elevated. The anticoagulant activity, purified from plasma by DEAE chromatography, was identified as a glycosaminoglycan (GAG). GAG anti-thrombin activity was completely abolished by heparin lyase III. Based on the degree of sulfation and HPLC pattern, the GAG was classified as heparan sulfate. Low levels (4 μM) of purified GAG markedly prolonged the TT (>120 s) but not the activated partial thromboplastin time (PTT) (31.4 s). In a Factor Xa assay, the GAG exhibited a potency equivalent to 0.06 U of low molecular weight heparin per nmol of uronic acid. Patients with endogenous circulating glycosaminoglycans can present with unusual laboratory coagulation test profiles. These reflect complex dysfunction of hemostasis, leading to difficulty in providing diagnosis and effective care. Am. J. Hematol. 58:285–292, 1998.

Key words: glycosaminoglycan; antithrombin III; heparin; thrombin time; partial thromboplastin time

INTRODUCTION

The coagulation system is regulated by a complex system of proteins and other biomolecules both in plasma and on the surface of cells. Dysregulation can have fatal consequences. We report a patient with cirrhosis and high levels of endogenous circulating heparan sulfate who developed a fatal coagulopathy. Syndromes involving circulating heparin-like anticoagulants (heparinoids) have been described [1], but have not been previously associated with hepatocellular carcinoma. We describe the primary structure of this anticoagulant, its effects on the coagulation system, and the special diagnostic and therapeutic problems presented by this type of endogenous anticoagulant.

METHODS

Clinical History

A 42-year-old man presented with cirrhosis, ascites, a liver mass, and a recent history of mucocutaneous bleeding. The patient had a past history of alcohol abuse and was anti-hepatitis C antibody positive. He was not taking

Laurence Corash's current address is Cerus Corporation, 2525 Stanwell Drive, Concord, CA 94520.

*Correspondence to: David S. Wages's current address: Cerus Corporation, 2525 Stanwell Drive, Suite 300, Concord, CA 94520.

Received for publication 10 April 1997; Accepted 8 April 1998

TABLE I. Initial Laboratory Coagulation Profile

	Patient	Reference range
PT (sec)	12.0	10.3–13.3
PTT (sec)	35.1	23.9–35.5
TT (sec)	99.9	24.0–35.0
Reptilase time (sec)	16.6	13.0–19.0
Platelet count ($\times 10^9$/liter)	200	140–450
Fibrinogen (g/liter)[a]	2.10	1.75–4.33
Bleeding time (min)	6	<11
Immunologic fibrinogen (g/liter)	3.30	2.28–5.01
Clauss fibrinogen (g/liter)[b]	2.57	1.75–4.33
D-dimers (mg/liter)	<0.5	<0.5
Factor XIII activity screen	Present	Present
Euglobulin lysis time (hr)	5.0	1.5–5.0
Antithrombin III antigen (mg/liter)[c]	1110	220–390
Antithrombin III (% activity)[c]	>500	84–123

[a]Functional, optical method.
[b]Fibrometer.
[c]Post mortem.

TABLE II. Characterization of the Inhibitor in a Thrombin Time Mixing Study*

	Thrombin time (incubation time, sec)	
Plasma	Immediate	60 Min
Patient	70.0	107
Control	32.1	29.1
Patient + control (1:1)	51.4	45.5
Fibrinogen deficient + control (1:1)	33.7	29.9

*Samples were incubated at 37°C for either 3 min (immediate) or 60 min before adding thrombin as described in Methods section.

TABLE III. Comparison of Human and Bovine Thrombin Time Assays*

Thrombin time (reference range 24–35 sec)			
Human thrombin		Bovine thrombin	
Normal plasma	27.1	Normal plasma	29.8
Patient plasma	107	Patient plasma	>120
Patient + toluidine blue	38.7	Patient + toluidine blue	38.9
		Patient + Hepabsorb®	39.7

*Hepabsorb®, a triethylaminoethyl resin (Organon Teknika, Durham, NC), in a final TT reaction concentration of 70 mg/ml in Tris saline (see Methods), or 0.1 ml of a 0.1% solution of toluidine blue in Tris saline instead of Tris saline, was added to make up the 0.3 ml TT reaction mixtures. Thrombin times using equivalent units of either human or bovine thrombin were done in a 0.3-ml final volume assay. Ortel et al. [2] reported thrombin times in the presence of anti-thrombin antibodies were corrected if human thrombin was used.

TABLE IV. Protamine Titration of Thrombin Time*

Final protamine concentration in thrombin time assay (μg/ml)	Thrombin time (sec; reference range: 24–35)
25	66.7
33	53.7
50	43.0
67	34.2
83	29.0

*Protamine sulfate in the indicated concentrations was added to the buffered saline solution used in the thrombin time reaction. Clinically, approximately 1 mg of protamine neutralizes 100 U of heparin [29]. Because the final concentration of protamine required to bring the TT into normal range was 67 μg/ml, this indicates the patient's plasma had the functional equivalent of approximately 6.7 U heparin/ml.

any medication. Fourteen months prior to admission the patient developed increasing abdominal girth with marked hepatomegaly, spider angiomata, and pruritis. Over the ensuing 6 months, he experienced easy bruising, epistaxis, and gingival bleeding. The patient had no history of a bleeding disorder and had undergone an appendectomy and partial colon resection for colitis in the past without incident. Four months prior to admission, he was clinically diagnosed as having cirrhosis. An abdominal computed tomography scan showed a 4 × 5 × 4 cm mass in the right lobe of the liver. The patient was subsequently referred to UCSF for liver transplant evaluation. Laboratory tests pertinent to this patient's history were performed (Table I). Despite his mucocutaneous bleeding history, only the thrombin time (TT) was abnormal.

A thrombin inhibitor was detected in a 1-hr mixing study (Table II). Because there was only partial correction in the mixing study, the prolongation could not be exclusively due to a factor deficiency. The possible presence of anti-bovine thrombin antibodies was evaluated by performing thrombin times using both human and bovine thrombin as described by Ortel et al. [2]. The TT was prolonged using either bovine or human thrombin and partially corrected with toluidine blue and a cationic resin (Hepabsorb®) (Table III). These results were considered inconsistent with an antithrombin antibody. Although the TT did not completely correct with the toluidine blue or Hepabsorb®, the partial correction and a protamine titration (Table IV) strongly suggested the presence of a heparin-like molecule.

The patient underwent a computed tomography-guided fine needle liver biopsy for cytologic examination of the hepatic mass; however, the biopsy was nondiagnostic. Over the ensuing 2 days he developed increasing abdominal pain accompanied by a slowly falling hemoglobin (from 9.2 to 6.4 g/dl). Abdominal ultrasound studies eventually confirmed the presence of a subhepatic hematoma. Protamine sulfate infusions of 200 mg intravenously every 4 hr did not shorten the TT. His coagulation parameters worsened as he developed signs of hepatic necrosis with resultant coagulopathy. A 5-liter plasma exchange reduced the TT to 35.7 sec temporarily, and the patient's subcapsular hematoma was surgically evacuated. At laparotomy, frozen sections showed numerous foci of well-differentiated hepatocellular carcinoma. After laparotomy, the TT increased again, and a

second 5-liter plasma exchange was performed, reducing the TT to 70.9 sec for approximately 6 hr, after which it markedly prolonged. Renal and hepatic function deteriorated rapidly, no further therapy was instituted, and the patient expired.

To further evaluate the greatly prolonged TT, antithrombin III measurements were performed using stored plasma. The results were markedly elevated (Table 1).

Isolation of Glycosaminoglycan (GAG) From Plasma

All reagents were from Sigma Co. (St. Louis, MO) unless otherwise noted. The exchanged plasma was stored in blood collection bags containing CPDA-1 (26.3 g/liter trisodium citrate, 3.27 g/liter citric acid, 31.9 g/liter dextrose, 2.22 g/liter monobasic sodium phosphate, and 0.275 g/liter adenine) at −70°C until chromatography. The purification procedure of Staprans and Felts was followed [3]. Briefly, the plasma was thawed, centrifuged at 4,000*g* to remove any cryoprecipitate, diluted 1:2 with 0.15 M NaCl, and applied to a 20 × 2 cm DEAE sephacel-ion-exchange column at room temperature. The column was washed with 3 volumes of 0.3 M NaCl. GAG-containing activity was eluted in 1.5 M NaCl and collected in 3-ml fractions. Fractions were analyzed for the presence of uronic acid, indicating the presence of GAG, and positive fractions were pooled [4]. The pooled fractions were dialyzed three times against 200 × volume of distilled, deionized H_2O at 4°C for a total of 24 hr using Spectrapor/6 dialysis tubing with a molecular weight restriction of 2000 (Spectrum Inc., Houston, TX). The dialyzed material was vacuum centrifuged or lyophilized, yielding a white to yellow powder (GAG fraction) that was highly soluble in water. Dissolved GAG-fraction in the appropriate buffer was used in subsequent assays. In all assays, the amount of GAG-fraction used in an assay was controlled by measuring the uronic acid concentration by the carbazole method [4].

Coagulation Assays

All coagulation assays were done in duplicate according to UCSF Laboratory Medicine Protocols according to National Committee for Clinical Laboratories Standards [5]. Thrombin times were performed in a final volume of 0.3 ml using 0.1 ml plasma, 0.1 ml Tris-saline (0.05M Tris Cl, pH 7.4, 0.56% NaCl) and 0.1 ml of 0.15 U/ml thrombin in Tris-saline. Normal control plasma and the reference range (mean ± 2 s.d.) were obtained using a plasma pool derived from 100 healthy donors. Thrombin time mixing studies (0.3 ml final volume) were performed using standard techniques and a reagent control consisting of fibrinogen deficient plasma (George King Co., Overland Park, KS) in equal parts with control plasma. Antithrombin III antigen was measured by fixed time nephelometry and activity was measured by a chromogenic assay (aca® discrete clinical analyzer, DuPont, Wilmington, DE). The GAG-fraction was dissolved in the same buffer used in control reactions. Control and experimental reactions were of equal volumes. Values represent the mean of an assay. PT and PTT assays were performed using Thromboplastin C Plus® and Actin® FSL reagents respectively (Baxter Diagnostic Inc., Deerfield, IL). Platelet aggregation was measured using a Chronolog 560VS whole blood aggregometer (Chronolog Co., Havertown, PA) as described [6]. The ristocetin cofactor assay was performed using reagents from Biodata Corporation (catalog 101246, Hatboro, PA). The GAG-fraction containing 25 nmol of uronic acid was used at a final concentration of 62.5 μM. This final concentration was 10× the concentration that caused prolongation of the TT to its assay limit. The Factor Xa assay was done using the Coatest Low Molecular Weight Heparin system (Chromogenix, Mölndal, Sweden) according to the manufacturer's instructions. The assay was done in the presence of GAG-fraction incorporated into the buffer containing 3 or 6 nmol of uronic acid (final concentration 7 and 14 μM).

The effect of the isolated GAG on thrombin-mediated platelet ATP release was determined by luminescence with modification of previously reported techniques [7,8]. Platelets washed free of plasma with Tyrodes Buffer (0.14 M NaCl, 5 mM EDTA, 0.5 mM NaH_2PO_4, 5 mM glucose, 1.2 mM $NaHCO_3$, 2 mM $MgCl_2$, 3 mM KCl, 0.5% BSA adjusted to pH 7.1 with NaOH) were resuspended to a concentration of 3×10^5/μl. Three hundred microliters of platelets were added to 0.5 ml glass cuvettes to which 100 μl of normal saline containing GAG with 0 to 20 nmol of uronic acid was added. After incubation for 15 min at room temperature, baseline luminescence was measured, followed by the addition of 25 μl chromogenic substrate (Chronolume, Chronolog Co). Thrombin was added and ATP release was measured as described [7,8]. The effect of the GAG on thrombin induced platelet secretion was measured by comparing the amount of ATP released in the absence and presence of the GAG fraction.

The effect of GAG on the platelet thrombin receptor was investigated by using the *Xenopus* oocyte thrombin receptor assay described by Vu et al. [9]. Briefly, *Xenopus* oocytes were microinjected with 12.5 ng of human wild type thrombin receptor mRNA and after expression was confirmed, the cells were incubated with [$^{45}Ca^{2}$+] and stimulated with the agonist thrombin, the peptide SFLLRN, and the antagonist Mpr in the presence of 50 nmol of uronic acid in the GAG-fraction (final concentration 100 μM).

The direct effect of GAG on thrombin activity was tested by a plasma fibrinopeptide assay manufactured by American Bioproducts Inc. (Parsippany, NJ). The manufacturer's instructions and the method of Liebman et al.

were followed [10]. The final uronic acid concentration in the reaction was 15 nmol/ml.

Characterization of Isolated GAG

GAG containing 15 nmol of uronic acid was digested using heparin lyases I and III and chondroitin lyase ABC as described [11]. The activity of the enzymes was assayed before use on the GAG samples by testing on pure substrates from Sigma and the concentration of the enzyme was adjusted to 0.5–1.8 mU/ml. All digestions were done with equal activities of enzyme. Proteinase K (Boehringer Mannheim, Indianapolis, IN) digestions of GAG samples were done using a final concentration of Proteinase K of 0.1 mg/ml for 8 hr at 37°C. The proteinase K was inactivated by heating samples in a boiling water bath for 10 min.

Disaccharide Analysis of the GAG

Preparation for analysis was as follows: lyophilized GAG fractions from DEAE-Sephacel column chromatography were pooled and treated with proteinase K (0.1 mg/ml) for 8 hr at 35°C. After heating at 100°C for 10 min samples were treated with $NaBH_4$ as described by Conrad [12]. The samples were treated with chondroitin ABC lyase as described earlier [11]. After heat treatment, samples were chromatographed over a 3-ml DEAE column, dialyzed against water as described above, and speed vacuumed. Disaccharide analysis was performed by Dr. Lowell Hager (Chirazyme Inc., Urbana-Champagne, IL) following the method of Guo and Conrad [13]. Briefly, GAG pooled from DEAE chromatography was treated with hydrazine and then nitrous acid to depolymerize the GAG into disaccharides. The depolymerized products were then labeled with [^{3}H] $NaBH_4$ to produce modified disaccharides with an anhydromannose replacing the glucosamine. These derivatives were then separated by either HPLC or paper chromatography for quantitative and qualitative analysis.

RESULTS

Partial Purification of Circulating GAG

Fractions eluted from the DEAE column were tested for the presence of uronic acid and for effects on the TT using normal plasma. The elution profile of uronic acid-containing fractions was identical to that described previously [3]. However, a prolongation effect on the TT was seen only from uronic-acid-containing fractions in the 1.5 M NaCl elution. In general, GAG-fractions containing between 1 to 2.7 nmol of uronic acid prolonged the TT to greater than 120 sec in a 0.3-ml assay.

Comparison of the Sensitivity of the PTT and TT to GAG Inhibition of Coagulation

Because the patient had a prolonged TT yet normal PTT, we explored the relative sensitivity of the two assays in the presence of the isolated GAG. While 1.2 nmol of uronic acid (final concentration 4 μM) in the GAG prolonged the TT to greater than 120 sec, the same amount had no effect on the PTT, which remained in normal range (Table V). The PTT was slightly prolonged in the presence of 1.6 nmol of GAG uronic acid, but in contrast to the TT, was not prolonged to its assay limit (100 sec) even in the presence of GAG containing 8 nmol of uronic acid (final concentration 26 μM).

TABLE V. Comparison of Sensitivity of the TT and the PTT to the GAG Inhibitor

Amount of GAG (nmol; final concentration μM)	Thrombin time (sec; reference range: 24–35)	Partial thromboplastin time (sec; reference range: 23.9–35.7)
0 (0)	23.5	30.6
1.2 (4.0)	>120	31.4
1.6 (5.3)		40.4
2.0 (6.7)		41.3
4.0 (13)		47.9
8.0 (26)		92.4

Enzymatic Characterization of the Circulating GAG

Polysaccharide lyases can be used to distinguish different GAGS [11]. The patient's isolated GAG was incubated with a panel of lyases followed by thrombin time assay to determine its structure based on lyase specific neutralization of the GAG inhibitor. Heparin lyase I cleaves glycosidic linkages in both heparin and heparan sulfate. However, the activity against heparan is reported to be only 13% of that against heparin [11]. In contrast, heparin lyase III acts only on linkages found in heparan sulfate. GAG fractions isolated from the patient's plasma were completely neutralized in the TT assay by heparin lyase III (Table VI). Treatment with heparin lyase I resulted in marked but still incomplete TT correction. Treatment with chondroitin lyase ABC, which acts endolytically on chondroitin and dermatan sulfates, did not affect GAG anticoagulant properties. Treatment of the GAG with proteinase K and heating to 100°C had no effect on GAG anti-thrombin activity (Table VI).

Disaccharide Analysis of the GAG as Heparan Sulfate

High performance liquid chromatography and paper chromatography revealed the GAG had only 51% of its disaccharides sulfated. Four sulfated species could be identified in the native GAG: iduronosyl 2-sulfate-glucosamine 2,6-disulfate, iduronosyl-glucosamine 2,6-disulfate, glucoronosyl-glucosamine 2,6-disulfate, and iduronosyl-sulfate-glucosamine 2-sulfate (Fig. 1). The unsulfated disaccharides of the GAG complex, iduronosyl-glucosamine and glucuronosyl-glucosamine, com-

TABLE VI. Effect of Enzyme Treatment on the Effect of the GAG on the Thrombin Time

Uronic acid concentration (μM)	Thrombin time (reference range: 24–35 sec)
0	24.6
90 (untreated)	>120
90 (heparin lyase I treated)	36.9
70 (heparin lyase I treated)	34.2
90 (heparin lyase III treated)	34.3
90 (chondroitin ABC lyase treated)	>120
90 (proteinase K treated)	>120
90 (heated at 100°C)	>120
0 (proteinase K treated, heated 100°C)	22.4
90 (proteinase K treated, heated 100°C)	>120

prise 21 and 28% of the GAG, respectively. While there are no definite characteristics that distinguish heparin from heparan sulfate, authentic heparin has an average sulfated glucosamine content of 85–95% [14]. Therefore, these findings strongly suggest that the isolated GAG is a heparan sulfate.

Effect of the GAG on Thrombin and Factor Xa

Liebman et al. demonstrated that a circulating GAG isolated from a patient with the heparinoid syndrome directly inhibited thrombin activity as measured by an assay for the split product of fibrinogen, fibrinopeptide A (FPA) [10]. We assayed for the release of FPA in the presence of our patient's isolated GAG and observed no difference between thrombin-mediated FPA production in the presence or absence of GAG (data not shown). This suggests that the GAG does not directly inhibit thrombin.

Activated ATIII inhibits both thrombin and activated Factor X. To determine if the GAG activity was limited to thrombin inhibition, we investigated its effects on Xa activity using a chromogenic substrate. In comparison to the effect of low molecular weight heparin standards, the GAG inhibited Factor Xa with a potency equivalent to 0.06 U of low molecular weight heparin/nmol uronic acid (Fig. 2). These data suggest that the GAG inhibits both activated Factor X and, indirectly, thrombin. These findings are consistent with ATIII mediated inhibition.

Effect of the GAG on Platelet Function

Because the patient had a history of both mucosal and delayed bleeding, we investigated the effect of the GAG on assays of in vitro platelet function. We examined thrombin-induced platelet ATP release in the presence of the GAG in response to various agonists. In these assays, the GAG had no consistent inhibitory or stimulatory effect on platelet function. In addition, we analyzed the effect of the GAG on von Willebrand factor activity since the von Willebrand factor contains a heparin binding site [15]. GAG fractions containing 25 nmol of uronic acid had no effect on ristocetin-induced von Willebrand factor activity.

Effect of the GAG on the Thrombin Receptor

To determine if the GAG could have exerted an antiplatelet effect by interfering with the thrombin receptor, we examined the effect of the GAG in the thrombin receptor system described by Vu et al. [9]. In this assay, thrombin receptor activation is monitored by measuring $^{45}Ca^{2}+$ release from oocytes expressing human thrombin receptors. The oocytes released labeled calcium in response to thrombin and the peptide agonist SFLLRN. Assay specificity was confirmed by blockade of $^{45}Ca^{2}+$ release when agonists were incubated in the presence of the inhibitor Mpr. When GAG fractions containing 50 nmol of uronic acid (final concentration 100 μM) were co-incubated with thrombin and SFLLRN, no inhibitory effect on the release of $Ca^{2}+$ was observed, suggesting the GAG did not affect thrombin receptor activity (data not shown).

DISCUSSION

Our investigation of this patient's coagulopathy raised several diagnostic and therapeutic issues in addition to providing further insight into the mechanisms of this type of coagulopathy. We demonstrated, for the first time by direct structural evidence, that a patient with heparinoid syndrome had increased circulating heparan sulfate. Heparin and heparan sulfate belong to the family of carbohydrates known as glycosoaminoglycans (GAGs). GAGs are complex macromolecules consisting of long chains of modified sugars and are present in the extracellular matrix and on the membrane surfaces of cells. The normal concentration in the plasma is very low [3]. Heparin and heparan sulfate are distinguished from other GAGs such as chondroitin sulfate by their monomer composition. Both heparan sulfate and heparin consist of repeating disaccharide units composed of uronic acid monomers (glucuronic or iduronic acid) and glucosamine. Each disaccharide can be further modified by sulfation. The different modifications and sequence of the disaccharide uronic-glucosamine repeat thus make up the primary structure of a heparin or heparan sulfate. Despite its name, heparan sulfate has fewer sulfated disaccharide units than heparin; however, there is no precise definition distinguishing heparan sulfate from heparin [14]. Purification of a completely homogeneous heparan sulfate or heparin is not possible because the sequence of the disaccharides is semi-random [12,16,17]. Thus, GAGs are distinguished on the basis of their monomers.

Heparin acts as an anticoagulant by binding and activating antithrombin III. ATIII in turn inhibits the coagulation cascade by binding thrombin, Factor Xa, and other

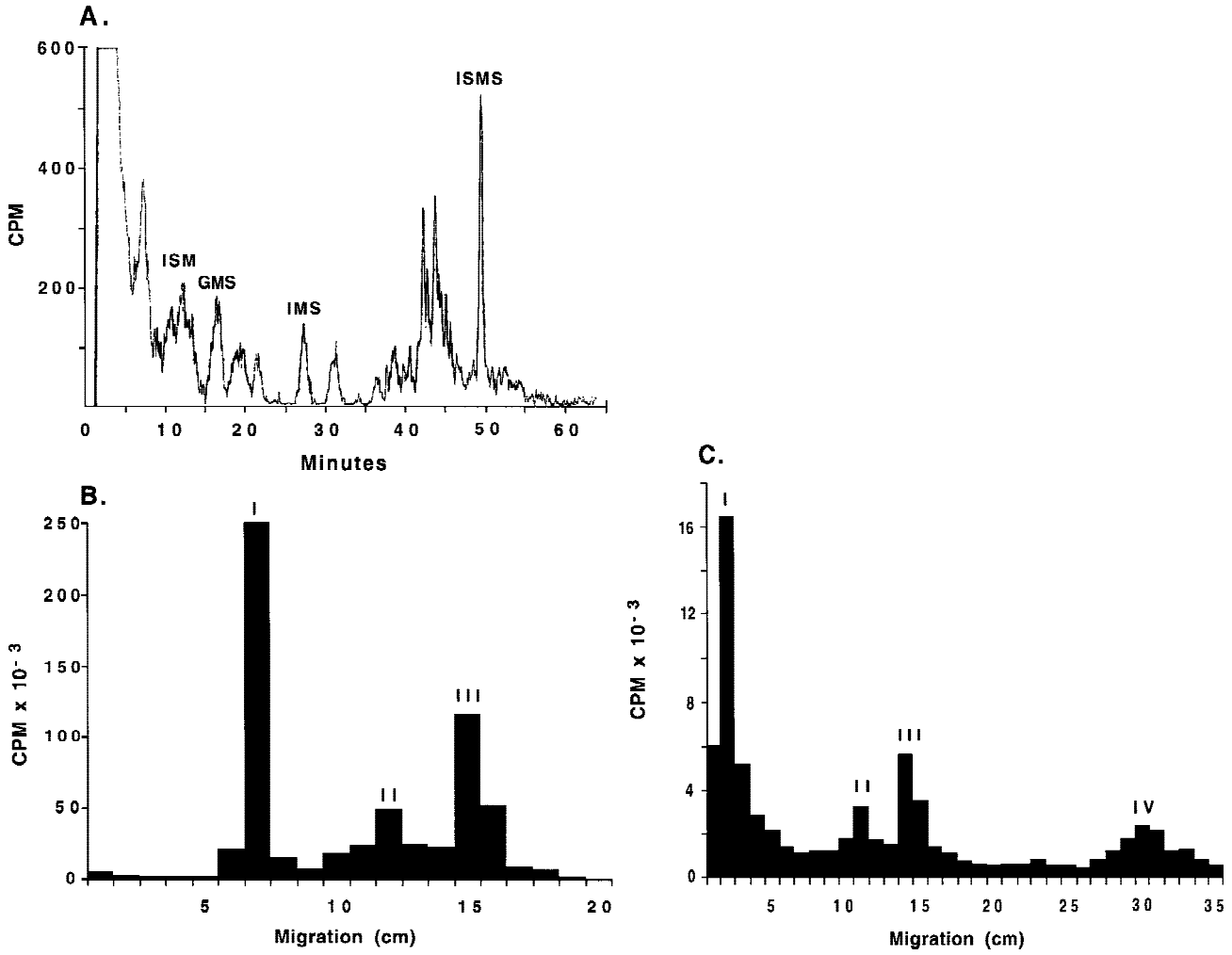

Fig. 1. Separation of [^{3}H]-labeled disaccharide species. A: Separation of disaccharides by HPLC. The modified products of hydrazinolysis of the GAG were separated by HPLC as described by Guo and Conrad [13]. ISMS, iduronosyl-sulfate-anhydromannose-sulfate; ISM, iduronosyl-sulfate anhydromannose; IMS, iduronosyl anhydromannose-sulfate; GMS, glucuronosyl anhydrommanose-sulfate. These derived products correspond to the following in native GAG: ISMS, iduronosyl-2-sulfate-glucosamine 2,6 disulfate; ISM, iduronosyl-2-sulfate-glucosamine 2-sulfate; IMS, iduronosyl-glucosamine 2,6 disulfate; GMS, glucuronosyl-glucosamine 2,6 disulfate. The relative amounts of these four identified species were 18:13:4:7. B: Paper electrophoresis of disaccharides. Peak I corresponds to unsulfated disaccharides while peaks II and III represent mono- and disulfated disaccharides, respectively. C: Separation of unsulfated disaccharides by paper chromatography. Peak I, total sulfated disaccharides; peak II, iduronic acid derivatives; peak III, glucuronic acid derivatives.

pro-coagulant serine proteases. Since heparin and heparan sulfate are expressed in a variety of cells, dysregulation of production could result in a bleeding diathesis similar to therapeutic heparin.

Heparinoid syndromes are rare and a collection of case reports has been reviewed [1]. Most patients with this syndrome have hematologic malignancies, but some have been reported in association with solid tumors [18,19]. In the few cases where the circulating heparinoid was indirectly and incompletely characterized, the majority have displayed characteristics consistent with heparan sulfate although dermatan sulfate has also been described [20]. There is also one reported case of heparinoid syndrome in a patient with systemic candidiasis and it has been reported in cancer patients who have received suramin [21,22]. Our patient represents the first case of heparinoid syndrome described in association with hepatocellular carcinoma. Presumably, the heparan sulfate was produced by the tumor; indeed hepatocyte cell lines have been demonstrated to produce heparan sulfate [23,24]. However, it would be difficult to distinguish tumor production of heparan sulfate from deposition from another source. For example, an alternative possibility is that a circulating enzyme may have cleaved heparan chains from endothelial cells lining the circulatory system.

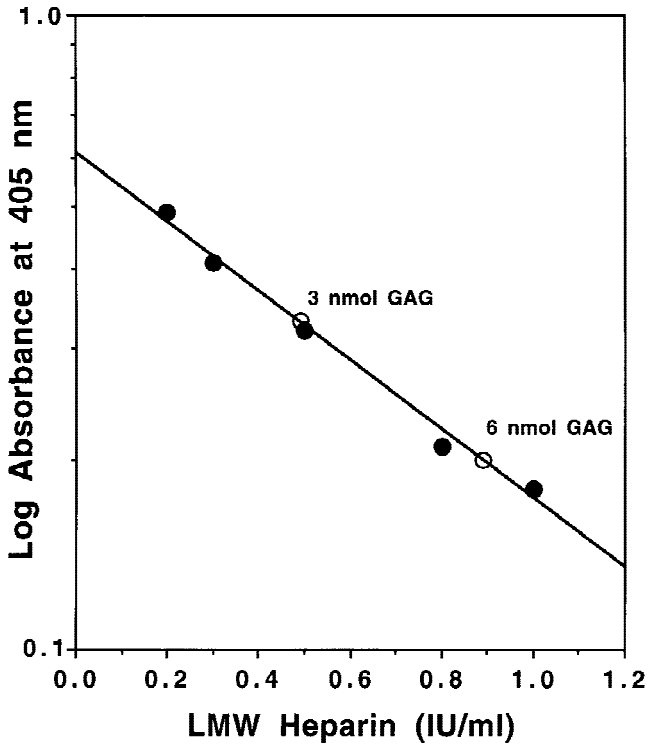

Fig. 2. Inhibition of Factor Xa by GAG compared to low molecular weight (LMW) heparin. LMW heparin (●) or GAG (○), was added to normal plasma along with S-2732, a chromogenic substrate of Factor Xa. When Factor Xa is added, it will either act on the substrate releasing a colored product measurable by absorbance or be inhibited by GAG/heparin-activated ATIII. Absorbance, thus, correlates directly with Factor Xa activity. Reactions were carried out for 8 min before being stopped with acetic acid. The final concentrations of the GAG-uronic acid were 7 and 14 μM. Increasing GAG is associated with diminished Factor Xa activity.

By protamine titration, the circulating heparan sulfate in our patient had an activity equivalent to 6–7 U/ml of heparin. In comparison, standard therapeutic anticoagulant levels are 0.2–0.4 U/ml [25]. The GAG in plasma obtained during plasmapheresis could not be accurately quantified due to dilution with allogeneic replacement plasma.

Our patient presented a diagnostic challenge because all routine coagulation assays were normal except the TT. Most patients with heparinoid syndrome are diagnosed by having prolonged activated partial thromboplastin times (PTT) or thrombin times (TT), which can be normalized by protamine sulfate. Only two of the 15 similar patients reported in the literature had normal PTTs, and only four had normal PTs, suggesting that the anticoagulant activity in the majority of cases is sufficient to affect the PTT and PT. The TT was prolonged in all patients in the literature. We were able to correlate the striking clinical difference between the patient's TT and PTT by demonstrating similar results with the column-purified GAG. This difference was seen despite a common anticoagulant mechanism involving ATIII binding. Reagents used in the PTT are variably sensitive to heparin; and a variety of factors are thought to be involved in this differential sensitivity [26]. Similar factors would likely apply to the thrombin time. The reason for this discrepancy remains unexplained.

The patient's markedly elevated ATIII was an unexpected finding as cirrhosis is usually associated with decreased levels of ATIII [27]. The patient's ATIII levels, higher than any reported, along with the circulating heparan sulfate may have acted in combination to produce the fatal coagulopathy. Elevated ATIII levels have been reported in patients with hepatocellular carcinoma without heparinoid syndrome [28], suggesting that elevated ATIII per se does not cause increased production of a circulating GAG. The patient's tumor may have independently manufactured excess amounts of both ATIII and heparan sulfate.

The severity of the patient's coagulopathy was demonstrated by its unresponsiveness to protamine sulfate therapy. The coagulation tests normalized only transiently after complete plasma exchange. The experience with this patient, and other reported cases in the literature [1], demonstrate that protamine sulfate cannot be completely relied upon as therapy in the heparinoid syndrome.

In summary, we describe the first case of a patient with heparinoid syndrome associated with hepatocellular carcinoma and elevated ATIII. This patient had a bleeding diathesis markedly out of proportion to his normal PT and PTT and there was no evidence of dysfibrinogenemia or platelet dysfunction, which could be expected in most patients with cirrhosis. The relative contribution of the elevated ATIII and the circulating heparan sulfate to the fatal hemorrhagic outcome remains undetermined.

ACKNOWLEDGMENTS

We thank Tom Lien and Agnes Frank for their technical assistance in preparing and testing the GAG, Andrew Connolly and Shaun Coughlin for the thrombin receptor assay, and Roger Lundblad for many helpful discussions. The authors are especially grateful to Lowell Hager and Chirazyme Inc., Urbana-Champagne, IL, for the disaccharide analysis.

REFERENCES

1. Tefferi A, Nichols WL, Bowie JW: Circulating heparin-like anticoagulants: Report of five consecutive cases and a review. Am J Med 88:184–188, 1990.
2. Ortel TL, Charles LA, Keller FG: Topical thrombin and acquired coagulation factor inhibitors: Clinical spectrum and laboratory diagnosis. Am J Hematol 45:128–135, 1994.

3. Staprans I, Felts JM: Isolation and characterization of glycosaminoglycans in human plasma. J Clin Invest 76:1984–1991, 1985.
4. Bitter T, Muir HM: A modified uronic acid carbazole reaction. Anal Biochem 4:330–334, 1962.
5. Lundberg G, ed: Collection, Transport and Processing of Blood Specimens for Coagulation Testing and Performance of Coagulation Assays. NCCLS Document H21-A2, 11(23), Ed. 2. Villanova, PA: National Committee for Clinical Laboratories Standards, 1991.
6. Macfarlane DE, Stibbe J, Kirby EP, Zucker MB, Grant RA, McPherson J: A method of assaying von Willebrand factor (ristocetin cofactor). Thromb Diath Haemorrh 34:306–308, 1975.
7. White MM, Foust JT, Mauer AM, Robertson JT, Jennings LK: Assessment of lumiaggregometry for research and clinical laboratories. Thromb Haemost 67:572–577, 1992.
8. Sheridan D, Carter C, Kelton JG: A diagnostic test for heparin-induced thrombocytopenia. Blood 67:27–30, 1988.
9. Vu TH, Hung D, Wheaton V, Coughlin SR: Molecular Cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. Cell 64:1057–1068, 1991.
10. Liebman HA, Comenzo R, Allen ST, Dilorio JM: A glycosaminoglycan inhibitor of thrombin: A new mechanism for abnormal hemostatic assays in cancer. Am J Hematol 38:24–29, 1991.
11. Linhardt RJ: Analysis of glycosaminoglycans with polysaccharide lyases. In Ausubel FM, Brent R, Kingston RE, et al. (eds): Current Protocols in Molecular Biology. New York: John Wiley & Sons, 1994, pp 17.13.17–17.13.32.
12. Conrad HE: b-elimination for the release of O-linked glycosaminoglycans from proteoglycans. In Ausubel FM, Brent R, Kingston RE, et al. (eds): Current Protocols in Molecular Biology. New York: John Wiley & Sons, 1995, pp 17.15.1–17.15.3.
13. Guo Y, Conrad HE: The disaccharide composition of heparins and heparan sulfates. Anal Biochem 176:98–104, 1989.
14. Conrad HE: Structure of heparan sulfate and dermatan sulfate. Ann NY Acad Sci 556:18–28, 1989.
15. Baruch D, Ajzenber N, Denis C, Legendre P, Lormeau JC, Meyer, D: Binding of heparin fractions to von Willebrand factor: Effect of molecular weight and affinity for antithrombin III. Thromb Haemost 71: 141–146, 1994.
16. Yuchuan G, Edward CH: Analysis of oligosaccharides from heparin by reversed-phase ion-pairing high-performance liquid chromatography. Anal Biochem 168:54–62, 1988.
17. Ornitz DM, Herr AB, Nilsson M, Westman J, Svahn C, Waksman G: FGF binding and FGF receptor activation by synthetic heparan-derived di-and trisaccharides. Science 268:432–436, 1995.
18. Rodgers GM, Corash L: Acquired heparin-like anticoagulant in a patient with metastatic breast carcinoma. West J Med 143:672–675, 1985.
19. Tefferi A, Owen BA, Nichols WL, Witzig TE, Owen WG: Isolation of a heparin-like anticoagulant from the plasma of a patient with metastatic bladder carcinoma. Blood 74:252–254, 1989.
20. Delorme MA, Saeed N, Sevcik A, Mitchell L, Berry L, Johnston M, Andrew M: Plasma dermatan sulfate proteoglycan in a patient on chronic hemodialysis. Blood 82:3380–3385, 1993.
21. Horne MK, Chao ES, Wilson OJ: Heparin-like anticoagulant associated with systemic candidiasis. Am J Hematol 35:37–42, 1990.
22. Horne MK, Stein CA, LaRocca RV, Myers CE: Circulating glycosaminoglycan anticoagulants associated with suramin treatment. Blood 71:273–279, 1988.
23. Bienkowski MJ, Conrad HE: Kinetics of proteoheparan sulfate synthesis, secretion, endocytosis and catabolism by a hepatocyte cell line. J Biol Chem 259:12989–12996, 1984.
24. Fedarko NS, Conrad HE: A unique heparan sulfate in the nuclei of hepatocytes: Structural changes with the growth state of cells. J Cell Biol 102:587–599, 1986.
25. Hirsh J: Heparin. N Engl J Med 324:1565–1574, 1991.
26. Bjornsson TD, Nash PV: Variability in heparin sensitivity of APTT reagents. Am J Clin Pathol 66:199–204, 1986.
27. Zalusky R, Furie B: Hematology in liver disease. In Hoffman R, Benz EJ, Shattil S, Furie B, Cohen HJ, Silberstein LE (eds): Hematology, Basic Principles and Practice. Ed 2. New York: Churchill Livingstone, 1995, pp 2096–2103.
28. Grieco A, De Stefano V, Cassano A, Ciabattoni A, Garufi C, Astone A, Leone G, Barone C: Hepatocarcinoma in cirrhosis: Is antithrombin III a neoplastic marker? Dig Dis Sci 36:990–992, 1991.
29. Physicians' Desk Reference. Ed 48. Montvale, NJ: Medical Economics Data Production Company, 1994, p 2537.